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Enumeration, Antimicrobial Resistance and Typing of *Salmonella enterica*: Profile of Strains Carried in the Intestinal Contents of Pigs at Slaughter in Southern Brazil

Daniel Santos Paim[1], Caroline Pissetti[1], Tatiana Regina Vieira[1], Gabriela Orosco Werlang[1], Eduardo de Freitas Costa[1], Jalusa Deon Kich[2] & Marisa Cardoso[1]

ABSTRACT

***Background*:** Despite a strong association between *Salmonella* isolation and slaughter hygiene, as measured by the *Enterobacteriaceae* levels on pre-chill carcass surfaces, a high variation in this association was observed between sampling days within the same slaughterhouse. It was hypothesised that in a scenario of high exposure on the farm, batches with a high prevalence of carrier pigs shedding a high number of *Salmonella* may enhance the risk of contamination on some slaughter days. Thus, the aim of this study was to assess the profile of *Salmonella* carried in the intestinal contents of slaughter pigs.
***Materials, Methods & Results*:** Ten pig batches slaughtered in a slaughterhouse were investigated for the presence of *Salmonella*. From each pig, the following samples were taken: *i*. blood collected at bleeding; *ii*. sponges rubbed on the carcass surface after bleeding and before chilling; *iii*. fragment of the ileocecal region of the intestine. Serum samples were subjected to a ELISA-Typhimurium test. Sponges were investigated for the presence of *Salmonella* and total aerobic mesophilic (TAM) and *Enterobacteriaceae* (EC) bacterial counts. *Salmonella* was enumerated in the intestinal contents. Selected *Salmonella* strains were subjected to an antimicrobial resistance disk diffusion test, macro-restriction with Xba-I (PFGE) and whole genome sequencing (WGS). From the 50 sampled pigs, 96% were positive in the ELISA-Typhimurium test and 64% were *Salmonella*-positive in the intestinal contents. The amount of *Salmonella* in the intestinal content samples was highly variable, and the mean log of fitted distributions of *Salmonella* in the batch ranged from -2.97 to 2.25 cfu.g^{-1}. The slaughter process achieved a logarithmic reduction, ranging from 0.64 to 2.35 log cfu.cm^{-2} for TAM and from 0.55 to 2.57 log cfu.cm^{-2} for EC. *Salmonella* was isolated from 16% of the carcasses after bleeding; this frequency decreased to 8% at the pre-chill step. All positive pre-chill carcasses originated from pigs carrying *Salmonella* in the intestinal content and from batches with a high number of carrier pigs. *Salmonella* Typhimurium and its monophasic variant were the most frequent in the intestinal contents and carcasses. Resistance was detected against ampicillin (42.5%), tetracycline (42.5%), sulfonamide (40%), gentamicin (25%) and ciprofloxacin (12.5%). Regarding colistin, 85% of the tested strains were classified as non-susceptible. The monophasic variant *S*. Typhimurium strains subjected to PFGE and WGS presented different profiles; several antimicrobial resistance genes were identified and all belonged to ST-19.
***Discussion*:** In this study, almost all sampled pigs entering the slaughter line had been exposed to *Salmonella* on the farm and a high number were carrying *Salmonella* in their guts. While the three batches with *Salmonella*-positive carcasses at the pre-chill step presented TAM media that was not significantly different from the other batches, there was a higher number of positive pigs carrying *Salmonella* in their intestinal contents. Moreover, the batch with the highest number of positive carcasses also presented the highest *Salmonella* mean count in their intestinal contents. The profile of *Salmonella* carried in the intestinal content of slaughter pigs proved to be highly variable in terms of the frequency, number of bacteria, serovars, antimicrobial resistance, and genotypes. Results indicate that the day-to-day variability in the prevalence and number of *Salmonella* in the intestinal contents of slaughter batches is likely to influence the frequency of contaminated pre-chill carcasses. *Salmonella* Typhimurium isolated from the intestinal contents of slaughter pigs may belong to genotypes involved in human disease and may carry several antimicrobial resistance genes. These aspects should be taken into account when planning *Salmonella* control in swine.

Keywords: *Salmonella* Typhimurium monophasic variant, faecal excretion, enterobacteria, total aerobic mesophilic bacteria, *mcr*-1 gene, whole genome sequencing.

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[1]Departamento de Medicina Veterinária Preventiva, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul (UFRGS). Porto Alegre, RS, Brazil. [2]Embrapa Swine and Poultry, Concórdia, SC, Brazil. CORRESPONDENCE: M. Cardoso [mcardoso@ufrgs.br - Tel.: +55 (51) 3308-6123]. Av. Bento Gonçalves n. 9090. Bairro Agronomia. CEP 91540-000 Porto Alegre, RS, Brazil.

INTRODUCTION

Salmonella control programmes targeting the poultry and swine production chains have been associated with a decreased prevalence of salmonellosis in humans [17]. In the European Union, the *Salmonella* control programme for swine has been based on the "from farm to fork" strategy, which includes on-farm interventions, the monitoring of *Salmonella* frequency in carcasses, and slaughter hygiene [1]. *Salmonella* control in the United States, in turn, is based mainly on improvements in slaughter hygiene; this approach has proved to be reliable and cost-effective [16]. In Brazil, a monitoring programme for slaughter hygiene and *Salmonella* frequency in pig carcasses has recently been published [8].

Previous studies conducted in southern Brazil demonstrated high *Salmonella* seroprevalence in slaughter pigs and a variable prevalence (from 8.7% to 24%) of *Salmonella* on pre-chill carcasses [15,25,42]. In Brazilian slaughterhouses, despite a strong association between *Salmonella* isolation and slaughter hygiene measured by the *Enterobacteriaceae* levels on pre-chill carcasses surfaces, a high variation in this association was observed between sampling days in any given slaughterhouse. It was hypothesised that in a scenario of high exposure on the farm, batches with a high prevalence of carrier pigs shedding a high number of *Salmonella* may enhance the hazard of contamination on some slaughter days. In this scenario, even a hygienic process would fail to avoid the presence of *Salmonella* on some carcasses [15]. Thus, the aim of this study was to assess the profile of *Salmonella* carried in the intestinal contents of slaughter pigs and the contamination of pre-chill carcasses.

MATERIALS AND METHODS

Study design and sampling

Ten pig batches slaughtered in a slaughterhouse located in Rio Grande do Sul were investigated for the presence of *Salmonella*. Pig batches were kept at the lairage for up to 8 hours; live animals were washed before entering the stunning and exsanguination area. The remaining slaughter steps were conducted as described by Silva *et al*. [43]. In order to minimise the effect of previous slaughter batches on the *Salmonella* environmental contamination, the first slaughtered batch for 10 consecutive days was sampled. From each batch, the fifth pig entering the slaughter line was included in the study and four other pigs, over an interval of 15 min, were also sampled. Pigs were marked in the left ear in order to be followed during the dressing process. From each pig, the following samples were taken: *i*. blood collected at bleeding; *ii*. sponges[1] rubbed on the carcass surface (400 cm^2) after bleeding and before chilling; *iii*. fragment of the ileocecal region of the intestine.

ELISA-Typhimurium

Serum samples were tested by the ELISA-Typhimurium test [27], with a cut-off value of 20% [26].

Total aerobic mesophilic (TAM) and Salmonella *isolation and enumeration by the most probable number (MPN)*

Sponges taken from the carcasses after bleeding and before chilling were individually suspended in 30 mL of 0.1% buffered peptone water and 5 mL of suspension was used for total aerobic mesophilic (TAM) and *Enterobacteriaceae* (EC) enumeration [43]. The TAM and EC results were calculated as $CFU.cm^{-2}$ and transformed into $\log_{10}$ for analysis. Means of $\log_{10}$ $CFU.cm^{-2}$ TAM and EC were compared by the Tukey test ($P = 0.05$) using SPSS software. The remaining suspension volume from each sponge was added to 225 mL of 1% buffered peptone water and subjected to *Salmonella* detection [24].

From the ileocecal region of the intestine, 25 g of intestinal contents were suspended in 225 mL of 1% buffered peptone water and subjected to *Salmonella* detection [24]. Intestinal contents were also subjected to *Salmonella* enumeration by the miniaturised most probable number (MPN) protocol described by Tavares [44], with modifications [34]. The MPN was calculated by the MPN Calculator (http://www.i2workout.com/mcuriale/mpn/index.html). Distrubutions of the concentrations of *Salmonella* sp. (log $cfu.g^{-1}$) in the sampled faeces were fitted using the maximum likelihood method [11] with the fitdistrplus package [18] of R software [39]. The method is suitable for estimating distributions with censored data, as is the case for MPN results and microbiological testing. In this model, the results of the screening test performed on the 25 g sample are considered together with the results of the MPN. In

this approach, a negative result in the screening is considered left censored (i.e. less than one bacterium in 25 g or less than 0.04 cfu.g^{-1}). Positive samples in the screening that were negative in the MPN are considered interval censorsed, while positive samples in the screening and in a finite number of MPN tubes are considered uncensored data. Finally, samples positive in the screening and presenting all positive MPN tubes are considered right censored data. *Salmonella* isolates were serotyped at the Fundação Instituto Oswaldo Cruz.

Antimicrobial resistance

All *Salmonella* strains were subjected to an antimicrobial susceptibility test against 12 antimicrobials by the disk-diffusion method, performed and interpreted by the Clinical and Laboratory Standards Institute, documents VET08 and M100 [13,14]. The following antimicrobials disks[2] were tested: ampicillin (10 µg), azithromycin (15 µg), cefotaxime (30 µg), cefoxitin (30 µg), ceftazidime (30 µg), ceftiofur (30 µg), ceftriaxone (30 µg), ciprofloxacin (5 µg), gentamicin (10 µg), meropenem (10 µg), sulfonamide (300 µg) and tetracycline (30 µg). Furthermore, the minimum inhibitory concentration (MIC) of colistin was also determined [21]. *Escherichia coli* ATCC® 25922 was used for quality control purposes. The strains were also screened for the presence of the *mcr*-1 gene by PCR with primers CLR5-F (5′-CGG TCA GTC CGT TTG TTC-3′) and CLR5-R (5′-CTT GGT CGG TCT GTA GGG-3′) [32].

Pulsed-field gel eletrophoresis (PFGE)

According to the serotyping profile, *Salmonella* strains were chosen to be subjected to pulsed-field gel eletrophoresis (PFGE) analysis, following the procedures of Pulse-Net (https://www.cdc.gov/pulsenet/pathogens/pfge.html). Isolates were digested with XbaI[3] and eletrophoresis was performed in a 1% agarose gel using 0.5X Tris-borate-EDTA buffer on a CHEF DR-II system[4]; this was done at 6 V/cm for 20 h at 14 ºC with an initial switch time of 2.2 s and a final switch time of 63.8 s. After PFGE, the gel was stained with ethidium bromide (2 µg/mL), photographed under UV transilumination and the image digitalisation was processed by the L-Pix Touch System[5]. PFGE-banding patterns were compared using the Gel-Compar II software package[6]. Similarities between profiles were calculated using the Dice coefficient, with 1.7% tolerance [12]. The patterns were clustered using the unweighted pair group method with arithmetic averages (UPGMA) and dendrograms were constructed.

Whole genome sequencing and multilocus sequence typing (MLST)

Chromosomal DNA of the subset of *Salmonella* strains was sequenced by MicrobesNG[7]. The genomic DNA was extracted using a PureLink Genomic DNA[8] kit, purified, resuspendend in EB buffer and quantified by QuantusTM Fluorometer dsDNA[3]. Genomic DNA libraries were prepared using a Nextera XT Library Prep Kit[9]. DNA quantification and library preparation were conducted on a Hamilton Microlab STAR Automated Liquid Handling System. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche LightCycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq[9] using a 250 bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cutoff of Q15 [5]. De novo assembly was performed on samples using SPAdes version 3.7 [3] and contigs were annotated using Prokka 1.11 [41]. The web-servers MLST version 2.0.1 [28] and ResFinder version 3.1 [47], available at the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org), were used to identify the multilocus sequence type (ST), chromosomal mutations and acquired antimicrobial resistance gene in genome with a selected threshold for 98% sameness. The identification and results of the ResFinder were compared with phenotypic antimicrobial susceptibility testing results. Serotyping was confirmed with SeqSero version 1.2, hosted by CGE [49].

RESULTS

From the 50 sampled pigs, 48 (96%) were positive in the ELISA-Typhimurium test, and 64% (32/50) of them were *Salmonella* positive in their intestinal contents. Pigs positive for *Salmonella* isolation were found in all 10 sampled batches, and the number of pigs carrying *Salmonella* per batch varied from one to five. The amount of *Salmonella* in the intestinal content samples of individual carrier pigs was highly variable, and the mean log of fitted distributions of *Salmonella* in the batch ranged from -2.97 cfu.g^{-1} (SD 6.8 cfu.g^{-1}) to 2.25 cfu.g^{-1}(SD 0.47 cfu.g^{-1}), as depicted in Figure 1.

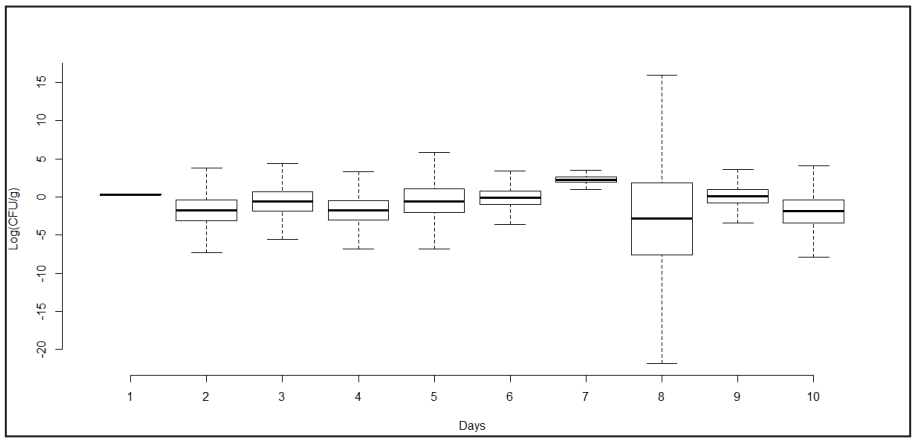


Figure 1. Fitted distribution of *Salmonella* sp. (log cfu.g-1) in the faeces of pig batches slaughtered over 10 different days.

The mean values of TAM and EC bacteria on the carcasses sampled after bleeding over 10 slaughter days varied from 2.64 to 3.58 log cfu.cm^{-2} and 0.27 to 2.64 log cfu.cm^{-2}, respectively (Tables 1 and 2). At the pre-chill step, the day-to-day mean of TAM bacteria ranged from 1.43 to 2.48 log cfu.cm^{2}, and the EC mean bacteria count ranged from -0.8 to 0.46 log cfu.cm^{2}. The logarithmic reduction achieved by the slaughter process varied from 0.64 to 2.35 log cfu.cm^{-2} for TAM bacteria and from 0.55 to 2.57 log cfu.cm^{-2} for EC. *Salmonella* was isolated from 16% (8/50) of the carcasses after bleeding; this frequency decreased to 8% (4/50) at the pre-chill step. Taking into account the four *Salmonella*-positive carcasses at the pre-chill step, two were originated from a common slaughter batch (#7), and the remaining two were from two different slaughter batches (#3 and #5). All positive pre-chill carcasses originated from pigs carrying *Salmonella* in their intestinal contents and from batches with a high number of carrier pigs (4/5 in batch #3 and 5/5 in batches #5 and #7).

Salmonella Typhimurium monophasic variant (S. 1,4,[5],12:-:1,2) was the most frequent serovar identified in the positive intestinal content samples (18/32; 56.25%). On the positive carcasses, *S*. Typhimurium predominated (5/12), followed by *S*. Derby (3/12) (Table 3).

Table 1. Mean logarithmic counts (log.cm^{-2}) of total aerobic mesophilic (TAM) bacteria on carcasses, after bleeding and before chilling, of pigs slaughtered in an abattoir in Rio Grande do Sul, Brazil.

Slaughter batch	Slaughter step		Mean logarithmic reduction
	After bleeding	Before chilling	
	Mean log TAM.cm^{-2} (Min. - Max.)	Mean log TAM. cm^{-2} (Min. - Max.)	
01	2.95 (2.67 - 3.36)^{cd}	2.02 (1.36 - 2.64)^{ab}	0.93
02	3.27 (2.90 - 3.45)^{bcd}	1.76 (1.26 - 1.97)^{ab}	1.51
03	4.25 (3.80 - 4.89)^{a}	2.48 (1.85 - 3.20)^{a}	1.77
04	3.09 (2.87 - 3.57)^{bcd}	1.44 (0.97 - 1.65)^{b}	1.65
05	3.78 (3.48 - 3.92)^{ab}	1.43 (1.08 - 2.03)^{b}	2.35
06	3.03 (2.72 - 4.03)^{cd}	1.86 (1.06 - 2.80)^{ab}	1.17
07	3.52 (2.78 - 3.92)^{abc}	1.85 (1.34 - 2.51)^{ab}	1.67
08	2.64 (2.00 - 3.27)^{d}	2.00 (1.72 - 2.37)^{ab}	0.64
09	3.35 (3.02 - 3.69)^{bcd}	1.79 (1.43 - 2.14)^{ab}	1.56
10	2.91 (2.79 - 3.13)^{cd}	1.63 (1.23 - 2.01)^{ab}	1.28

Different superscript letters in a row indicate significantly different means, according to a Tukey test ($P < 0.05$).

Table 2. Mean logarithmic counts (log.cm^{-2}) of *Enterobacteriaceae* (EC) bacteria on carcasses, after bleeding and before chilling, of pigs slaughtered in an abattoir in Rio Grande do Sul, Brazil.

Slaughter batch	Slaughter step		Mean logarithmic reduction
	After bleeding	Before chilling	
	Mean log EC.cm^{-2} (Min. - Max.)	Mean log EC.cm^{-2} (Min. - Max.)	
01	0.80 (ND* - 1.00)*	-0.71 (ND - -0.30)	1.51
02	1.33 (0.57 - 2.41)	-0.50 (-1.00 - 0.00)	1.83
03	2.64 (2.00 - 3.04)	0.23 (-0.15 - 0.77)	2.41
04	0.93 (0.38 - 1.63)	-0.80 (ND - -0.70)	1.73
05	2.16 (1.64 - 3.05)	-0.41 (-1.00 - 0.18)	2.57
06	1.45 (0.32 - 3.05)	0.46 (ND - 1.03)	0.99
07	1.18 (1.02 - 1.42)	-0.42 (-0.70 - 0.20)	1.60
08	0.27 (-1.00 - 1.12)	-0.28 (-0.70 - -0.05)	0.55
09	1.12 (0.11 - 1.98)	-0.34 (ND - -0.22)	1.46
10	0.45 (0.00 - 0.96)	-0.22 (-1.00 - 0.32)	0.68

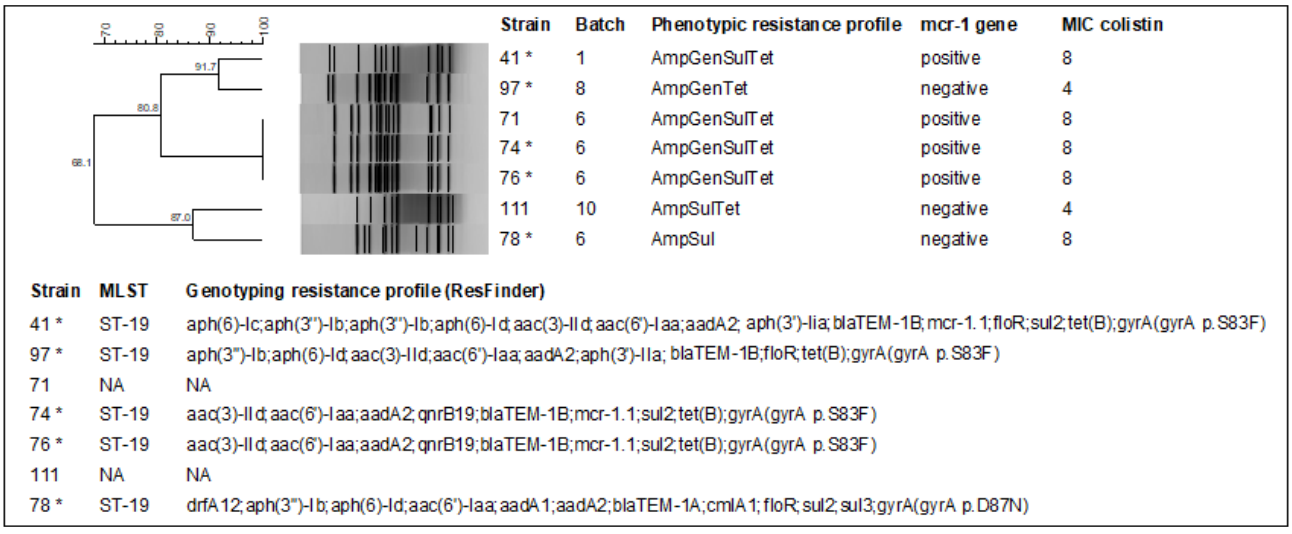
*ND (not detected): below the detection power of the methodology (10 cfu.cm^{-2}).

Table 3. Serovars of *Salmonella enterica* isolated from intestinal contents and pig carcasses sampled after bleeding and before chilling in a slaughterhouse in Rio Grande do Sul, Brazil.

Slaughter batch	Intestinal contents (n. positive samples)	Carcasses (n. positive samples)	
		After bleeding	Before chilling
01	Typhimurium monophasic variant (2)	Derby (1)	-
02	Mbandaka (1)	-	-
03	Typhimurium monophasic variant (4)	-	Derby (1)
04	Infantis (1)	-	-
05	Typhimurium monophasic variant (2); Derby (1); O:4,5 (1)	Typhimurium (1); Derby (1); Brandenburg (1)	O:4,5 (1)
06	Typhimurium monophasic variant (4)	Typhimurium (1)	-
07	Typhimurium monophasic variant (3); Typhimurium (1); Panama (1)	Typhimurium (2); Panama (1)	Typhimurium (1); Infantis (1)
08	Typhimurium monophasic variant (2)	-	-
09	Panama (2); Infantis (1)	-	-
10	Typhimurium monophasic variant (1); Infantis (1)	-	-

All the tested *Salmonella* strains isolated from intestinal contents (n = 28) and carcasses (n = 12) were fully susceptible to azithromycin, cephalosporins (cefotaxime, cefoxitin, ceftazidime, ceftiofur, and ceftriaxone) and meropenem. Resistance was detected against ampicillin (n = 17; 42.5%), tetracycline (n = 17; 42.5%), sulfonamide (n = 16; 40%), gentamicin (n = 10; 25%), and ciprofloxacin (n = 5; 12.5%). A total of six strains (15%) were susceptible to all tested antimicrobials and 17 (42.5%) were multi-drug resistant (MDR) strains (resistant to ≥ 3 antimicrobial classes). Regarding colistin, 85% (34/40) of the tested strains were classified as non-susceptible (MIC > 2 μg.mL^{-1}). In four of the non-susceptible strains (4/34; 11.8%), the gene *mcr*-1 was detected; all positive strains were *S*. Typhimurium monophasic variant and presented a MDR profile. In all *mcr*-1-positive strains, the MIC value for colistin was 8 μg.mL^{-1}; however, in one strain presenting this MIC, the *mcr*-1 gene was not detected.

The seven monophasic variant *S*. Typhimurium strains subjected to PFGE presented five different profiles; the pulsotype SM-1 encompassed three strains isolated from a common slaughter batch, presenting a common antimicrobial resistance profile and carrying the *mcr*-1 gene. The remaining PFGE pulsotypes included single strains with variable resistance profiles and originated from different pig batches (Figure 2).



Strain	Batch	Phenotypic resistance profile	mcr-1 gene	MIC colistin
41 *	1	AmpGenSulTet	positive	8
97 *	8	AmpGenTet	negative	4
71	6	AmpGenSulTet	positive	8
74 *	6	AmpGenSulTet	positive	8
76 *	6	AmpGenSulTet	positive	8
111	10	AmpSulTet	negative	4
78 *	6	AmpSul	negative	8

Strain	MLST	Genotyping resistance profile (ResFinder)
41 *	ST-19	aph(6)-Ic;aph(3'')-Ib;aph(3'')-Ib;aph(6)-Id;aac(3)-IId;aac(6')-Iaa;aadA2; aph(3')-IIa;blaTEM-1B;mcr-1.1;floR;sul2;tet(B);gyrA(gyrA p.S83F)
97 *	ST-19	aph(3'')-Ib;aph(6)-Id;aac(3)-IId;aac(6')-Iaa;aadA2;aph(3')-IIa; blaTEM-1B;floR;tet(B);gyrA(gyrA p.S83F)
71	NA	NA
74 *	ST-19	aac(3)-IId;aac(6')-Iaa;aadA2;qnrB19;blaTEM-1B;mcr-1.1;sul2;tet(B);gyrA(gyrA p.S83F)
76 *	ST-19	aac(3)-IId;aac(6')-Iaa;aadA2;qnrB19;blaTEM-1B;mcr-1.1;sul2;tet(B);gyrA(gyrA p.S83F)
111	NA	NA
78 *	ST-19	drfA12;aph(3'')-Ib;aph(6)-Id;aac(6')-Iaa;aadA1;aadA2;blaTEM-1A;cmlA1;floR;sul2;sul3;gyrA(gyrA p.D87N)

Figure 2. (A) Macro restriction profiles, antimicrobial resistance, amplification of *mcr*-1 gene and colistin minimum inhibitory concentration (MIC) of the *Salmonella* Typhimurium monophasic variant isolated from the intestinal contents of slaughtered pigs. (B) Multilocus sequence type (MLST) and resistance gene profile of strains subjected to whole genome sequencing (marked with an asterisk). Amp = ampicillin; Gen = gentamicin; Sul = sulfonamide; Tet = tetracycline; NA = non-applicable.

In order to further characterise the monophasic variant of *Salmonella* non-susceptible to colistin, five strains, which are indicated with an asterisk in Figure 2, were subjected to whole genome sequencing (WGS). The two strains (#74 and #76) belonging to the same pulsotype proved to carry the same genes conferring resistance to aminoglycosides, ampicillin, sulfonamide and tetracycline. Both strains presented the same mutation in the genes *gyrA* and carried *qnrB*; however, in the disk diffusion test, they were classified as intermediately resistant to ciprofloxacin. Colistin resistance was related to the presence of *mcr*-1. Strain #78, although isolated from this same slaughter-batch and presenting a MIC of 8 $\mu g.mL^{-1}$, did not carry any *mcr* gene and the resistance gene profile was distinct. In this strain, additional aminoglycoside resistance genes (*aad* and *aph*) were present, as well as genes codifying resistance against trimethoprim (*drfA12*) and the amphenicol class (*cmlA1* and *floR*). Strain #41, which was isolated on a different slaughter day but had the *mcr*-1 gene, as detected by PCR, also presented the *mcr1*.1 variant. Moreover, this strain presented the highest number of resistance genes (13 genes as well as the mutation on *gyrA*), and carried a large diversity of *aph* genes conferring resistance to aminoglycosides. Strain #97 grouped close to the latter in PFGE and was isolated on a different slaughter day; it presented a colistin MIC of 4 $\mu g.mL^{-1}$ and was negative in the *mcr*-1 amplification. WGS confirmed that no *mcr* gene type was present in this strain, while genes codifying resistance to ampicillin, aminoglycosides, amphenicol and tetracycline were detected. In spite of the diversity in the resistance genes, all strains were classified in a common sequence type (ST), namely ST-19.

DISCUSSION

In this study, almost all sampled pigs entering the slaughter line were seropositive in the ELISA test, demonstrating that they had been exposed to *Salmonella* on the farm. Moreover, a high number of the sampled pigs were carrying *Salmonella* in their guts. Serology gives historical information [26,27], thus a positive result in the ELISA-Typhimurium test cannot be interpreted as an active infection at slaughter, but indicates that the pig was infected on the farm. However, seropositive pig batches have higher chances of including carrier individuals, which may be excreting *Salmonella* at slaughter [4,7,40]. In fact, in the 10 sampled slaughter batches, at least one pig was carrying *Salmonella* in the gut, demonstrating a continuous source of contamination to the slaughter process represented by seropositive pig batches. In Brazil, a high prevalence of *Salmonella* carrier pigs at slaughter has already been reported [25,42], which may turn out to be a bottle neck in the control of *Salmonella* in pork.

The high burden of *Salmonella*-carrier pigs to the slaughter process makes hygiene of utmost importance, and avoiding contact of the carcass with intestinal contents and the environment contaminated

with faeces is key for maintaining the carcass status. The hygienic measures, in turn, should begin before slaughter; it has been demonstrated that lower total bacteria counts were found on the body surface of pigs that were washed before slaughter [36]. This suggests that washing may contribute not only to visibly clean animals [7], but also to lower bacterial counts on carcasses at the beginning of the process. In this study, TAM mean bacteria counts below 4 log cfu.cm^{-2} were detected in all batches, much lower than that reported (6 log cfu.cm^{-2}) by Pearce *et al*. [36] when washing was not performed.

In the slaughter process, some steps, such as singeing, can decrease superficial microbial contamination, while others represent a hazard of adding bacteria to the surface of the carcass [7,10,36]. The monitoring of hygiene indicators, such as TAM and EC, plays a role in reflecting the contamination/decontamination profile throughout processing. In fact, these results demonstrate that the process in place at the studied slaughterhouse was able to achieve logarithmic reductions of up to 2.5 log for TAM and EC bacteria counts. At the end of the slaughter line, mean counts of TAM and EC were below 3 log cfu.cm^{-2} and 1 log cfu.cm^{-2}, respectively. These mean counts are in accordance with those reported in studies conducted in slaughterhouses with hygienic process measures [19,36,50], and they are in compliance with the limits established in Brazil [8]. Therefore, on all slaughter days, the process proved to be in accordance with good hygienic standards.

Regarding *Salmonella*, slaughter processing was able to halve the number of positive carcasses detected after bleeding, despite 64% of the sampled pigs carrying *Salmonella* in their guts. A frequency of 8% *Salmonella*-positive carcasses found at the pre-chill step was reported in previous studies conducted in Brazil [15,25] and is in accordance with the prevalence estimated by a national exploratory study conducted by Brasileiro *et al*. [9]. However, the day-to-day variation was similar to previously reported variations [15]. *Salmonella*-carrier pigs are recognised as the main source of contamination at slaughter, and the number of pigs excreting *Salmonella* is an important factor for carcass contamination [4,10,17]. Still, data about the number of *Salmonella* carried in the intestinal contents of pigs at slaughter and its relationship with the carcass status are scarce. In a study conducted in the Netherlands, the mean number of *Salmonella* carried in pig rectums at slaughter varied from -3.32 to 2.71 log MPN.g^{-1} over 11 sampling days, demonstrating high variability in the number of *Salmonella* excreted by pigs [45]. In the aforementioned study, however, the influence of this variation on the frequency of *Salmonella* in carcasses was not investigated. While in our study the three batches with *Salmonella*-positive carcasses at the pre-chill step presented TAM media that was not significantly different from the other batches, there was a higher number of positive pigs carrying *Salmonella* in their intestinal contents. Moreover, the batch with the highest number of positive carcasses also presented the highest *Salmonella* mean count (2.25 log cfu.g^{-1}) in the intestinal contents of the pigs. Although the *Salmonella* strains detected in the intestinal contents and positive carcasses belonged to different variants or serovars, the burden of the number of *Salmonella* in the gut to the slaughter process can be inferred. Moreover, it was demonstrated that *S*. Typhimurium and its monophasic variant predominated in both carcass and intestinal content samples. Over the years, *Salmonella* Typhimurium has been the most prevalent serovar reported in swine in Brazil [25,42]. In recent years, the monophasic variant of serovar Typhimurium (*S*. 1,4,[5],12:-:1,2) has started to be described in subclinically-infected pigs or pigs with diarrhea [20,33]. This serovar and its monophasic variant are highly pathogenic and often carry antimicrobial resistance genes, and are thus a relevant hazard to consumers [6].

The antimicrobial resistance profile of *Salmonella* has become an important topic in public health, since the selection of resistant strains on the farm can reach humans through the food chain and the environment [46]. While the tested strains were fully susceptible to all tested cephalosporins, indicating that extended beta lactamases producer strains were absent, a high percentage of MDR strains were identified, mainly presenting a resistance profile to ampicillin-tetracycline-sulfonamide. This profile is the most prevalent in studies reporting antimicrobial resistance in *Salmonella* isolated from swine [20,30]. Considering the hazard for humans though the resistance to the 3rd and 4th generations cephalosporins and fluoroquinolones are critically important, since they are the antibiotics that are used

for the treatment of invasive salmonellosis or infections in children [46]. In this regard, the resistance to ciprofloxacin, which was found in 12.5% of the strains, is of concern.

In recent years, resistance to colistin, codified by genes *mcr* carried in transferable plasmids, has been increasingly reported [31,48]. This is a matter of concern since colistin is considered the last resort drug, which is used for the treatment of human infections caused by bacteria resistant to all other antimicrobial classes. Most of the tested *Salmonella* strains (85%) were inhibited by colistin concentrations > 2 μg.mL^{-1} and thus, were considered non-susceptible according to the only available criteria [21]. Colistin resistance can be caused by chromosomal mutations or by the *mcr* gene carried on plasmids. The latter can in turn be transferred horizontally amongst the bacterial population, allowing for the rapid shift for resistance [31,32]. The investigation of the *mcr*-1 gene among the *Salmonella* strains demonstrated that only 11.8% of the non-susceptible strains carried this gene. Although *mcr*-1 was the first colistin resistance gene to be identified, to date, five different *mcr* and their variants have been described [48]. Among them, *mcr*-1, *mcr*-2 and *mcr*-3 were described on plasmids carried by Enterobacteriaceae, and *mcr*-1 and *mcr*-2 proved to be highly prevalent in samples taken from swine in China [31,48]. Although in our study *mcr*-1.1 was the only variant identified in the strains subjected to WGS, other gene variants could be present in the other strains and could have been responsible for the observed MIC ≥ 4 μg.mL-1 profile.

All *mcr*-1-positive strains by PCR were identified as monophasic variants of *S*. Typhimurium isolated from intestinal contents; three presented a common resistance profile, were isolated from the same pig batch and their PFGE profiles were indistinguishable. These characteristics indicate that they might belong to a resistant clone carried in the gut of the pigs from this batch. This hypothesis was supported by WGS, which demonstrated a common profile of resistance genes in the strains.

All PFGE closely-related strains subjected to WGS shared several resistance genes: *aadA2* (streptomycin resistance); *aac(6')Iaa* (amikacin and gentamicin resistance) and $bla_{\text{TEM-1B}}$ (ampicillin resistance). All of these genes are widely distributed in *Salmonella* and other enterobacteria and are usually part of MDR profiles [22,29,30,37]. Additional genes commonly reported in *Salmonella*, such as *tetB* (tetracycline resistance), *sul2* (sulfonamide resistance) and *floR* (amphenicol resistance), were also found in most of the strains. Regarding the fluoroquinolones, the five strains were phenotypically not resistant to ciprofloxacin, but carried mutations in *gyrA*, which confers resistance to nalidixic acid and reduces susceptibility to fluoroquinolones [2,38]. In two strains (#74 and #76), which were phenotypically intermediately resistant to ciprofloxacin, the plasmidial gene *qnrB19* was also present. This gene also confers low resistance to fluoroquinolones and facilitates the development of mutations in *gyrA* [38]. All these findings indicate that these strains are already not fully susceptible to this antimicrobial class. This result together with the phenotypically-resistant strains detected in the disk diffusion test highlight the importance of the use restriction of this antimicrobial class to only therapeutic purposes in swine.

Besides the resistance profile identification, WGS allowed us to classify the monophasic variant *S*. Typhimurium strains into a sequence type (ST-19) frequently involved in foodborne outbreaks, including several in which pork was the likely source [23]. Moreover, ST-19 was likely to be the most frequent among *Salmonella* strains isolated from human infections in Brazil [35]. This constitutes an additional hazard for *Salmonella* control in pork, and demonstrates that resistant and pathogenic *Salmonella* strains may be continuously introduced in the slaughter process by pigs infected on the farm.

CONCLUSIONS

The profile of *Salmonella* carried in the intestinal contents of slaughter pigs is highly variable in terms of the frequency, number of bacteria, serovars, antimicrobial resistance and genotypes. Results indicate that the day-to-day variability in the prevalence and number of *Salmonella* in the intestinal contents of slaughter batches is likely to influence the frequency of contaminated pre-chill carcasses. *Salmonella* Typhimurium, isolated from the intestinal contents of slaughter pigs, may belong to genotypes involved in human disease and may carry several antimicrobial resistance genes. These aspects should be taken into account when planning the control of *Salmonella* in swine.

MANUFACTURERS

[1]Nasco. Fort Atkinson, WI, USA.

[2]Oxoid Ltd. Hampshire, UK.

[3]Promega Biotecnogia do Brasil. São Paulo, SP, Brazil.

[4]Bio-Rad. Hercules, CA, USA.

[5]Loccus Biotecnologia. Cotia, SP, Brazil.

[6]Applied Maths. Kortrijk, Belgium.

[7]MicrobesNG. Birmingham, UK.

[8]Invitrogen Life Technologies. Carlsbad, CA, USA.

[9]Illumina Inc. San Diego, CA, USA.

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